Musculoskeletal Research Center

Summer Research Program

2009

Department of Bioengineering

University of Pittsburgh

MSRC

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2009 Abstract Book Committee

This summer, undergraduate students from across the country took part in an exciting and unique research experience here at University of Pittsburgh's Musculoskeletal Research Center. We all came from different educational backgrounds, but for most part, most of us did not know what exactly to expect. Soon enough however, we strived to form a great relationship with our fellow co-workers, mentors, and students, and learned valuable lessons along the way. These past ten weeks might have been the fastest of any summer, and our thanks go out to the faculty here that made our projects possible. Without their guidance and expertise, it's safe to say we would be completely and utterly lost. This book represents is a compilation of all our, as well as a bit of history about ourselves, and our interests in the field of bioengineering. Once again, we'd like to thank the entire MSRC for this opportunity and a wonderful summer!

Gautam Vangiupram
Abstract Book Committee

2009 Summer Symposium Committee

This year the Summer Student Symposium took place on July 23,2009 at the in the Thermofischer Scientific Board Room. Each of the fourteen summer students were given the opportunity to present what they had completed during their 10 week internship and the opportunity to answer questions from MSRC Staff and other faculty from the University. Even though nerves were tight and little sleep was gotten the night before each student was able to give amazing presentations and each student eloquently answered questions posed by the viewers. Yet, not only was each student able to majestically present all of their hard work and answer difficult questions but each of us will walk away with skills which will prove worthy in upcoming careers. On behalf of the symposium committee I would like to thank all of those who helped us make this year's symposium a success, as well all of those who attended the symposium. I would also like to give a special thanks to Dr. Woo, for the wonderful opportunity this summer research experience gave us all, and Dr. Debski for all of his work in making this year's summer research program a success.

Brooklynn Rowland
Chair, Summer Symposium Committee

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I was born on November 9th 1987 in Langhorne, P.A. and spent the first few years of my life in Fairless Hills, P.A. Then in July of 1993 my family moved to Greensburg, P.A. and began life in the Pittsburgh area. I am the oldest of three children and graduated from Hempfield Area High School in 2006. While in high school I participated in a variety of activities included football, track, NHS, and played the saxophone in the jazz and marching bands….yes marching band and football can be done together.

Outside of class I enjoy spending time going to all types of Pittsburgh/Pitt sporting events as well as exploring the culture and history that Pittsburgh has to offer. When choosing a career path I found bioengineering to combine both my curiosity for how and why things work with an interest in sports medicine ultimately. I found Pitt to be a very attractive program considering its access to world class hospitals and their focus on undergraduate research. While my focus has shifted from sports medicine, my passion for learning and improving medicine has been reinforced and propelled forward during my time at the University of Pittsburgh.

My experience at the MSRC has been remarkable. It has been a privilege to work such an outstanding group of individuals who truly care about the research they are performing. This experience has significantly advanced my academic career and given me a better grasp on what I would like to accomplish in my future endeavors. I would like to thank Andrew Feola and Dr. Abramowitch for all of their help and guidance and Dr. Woo for an incredible summer at the MSRC.

THE IMPACT OF PREGNANCY ON THE VISCOELASTIC BEHAVIOR OF THE RAT UTERINE CERVIX IN UNCONFINED COMPRESSION

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SUMMARY

Preterm labor is the leading cause of neonatal mortality and accounts for 70% of the total cost of neonatal health care. One of the primary causes of preterm labor is premature softening of the cervix. Understanding the properties cervix will allow for the pathophysiology of preterm labor to be evaluated. The objective of this study was to determine the viscoelastic properties of virgin (n=5), pregnant (14-15 days, n=4), and late post partum (n=4) Long-Evans rat cervical tissue. The cervix was placed in a saline solution at room temperature, and tested using an unconfined compression protocol that included 3 trials of a ramp and hold (4 min) test to 20% strain. The tissue response was modeled using the quasi-linear viscoelastic (QLV) theory developed by Professor Fung (1972). The parameters governing the elastic response, A and $A \times B$, were found to be significantly different between virgin and postpartum cervix ($p<0.05$). These differences result in a greater slope of the elastic stress-strain curve showing a stiffer response for postpartum tissues. The change in post-partum tissue may reflect a pregnancy induced remodeling process that has not fully recovered back to virgin levels or, more likely, a healing response which results in a stiffer tissue. Future studies will evaluate the cervix later in gestation, perform biochemical assays, and validate QLV constants by predicting experimental results with a different strain history.

DESCRIPTION OF DESIGN OBJECTIVE

Preterm labor is a serious event which occurs in up to 12% of all pregnancies in the United States and is the leading cause of neonatal mortality in America [1]. As a result, 70% of all money spent on neonatal health care is due to preterm delivery caused by preterm labor [1]. The main cause of preterm labor is the premature softening of the cervix [2]. The cervix is a structure within the female reproductive tract which bridges the uterus to the vagina. Throughout pregnancy the cervix undergoes a number of large transformations. A process known as "ripening" causes the cervical collagen structure to become disrupted, muscle tissue to undergo apoptosis, and water concentration to increase [2]. Each of these changes occurs progressively, allowing the cervix to become a softer structure as the fetus approaches delivery. Ideally the cervix retains sufficient mechanical stiffness and strength to retain the fetus until term is reached. However, if the cervix ripens before term, it is a major cause of premature delivery. In order to understand preterm labor and design an effective clinical test for preterm labor prediction the cervix must be analyzed as a mechanical structure [3]. By examining the viscoelastic properties of the cervix we can predict its behavior to loading conditions. Like the human, the cervix of the rodent model is largely comprised of collagen, gylcoaminoglycans, smooth muscle, elastin, and water. Also the cervix of the rat anatomically mimics the human cervix in that both connect the uterus to the vagina and retain the fetus during pregnancy [2].

The long-term goal of this research is to evaluate the rat as a model to study the mechanics of cervical ripening. As a first step toward this goal, the objective of this study was to use unconfined compression testing in order to determine the viscoelastic properties of virgin, pregnant, and post partum Long-Evans rat cervical tissue. Differences in the viscoelastic behavior between the groups will help to define the changes that occur throughout and after pregnancy, and the underlying causes of preterm labor. Based on literature that shows increased water content and structural alterations in the cervix throughout pregnancy, we hypothesize that the pregnant tissue will display a less stiff elastic response with increased viscous behavior compared to virgin tissue. In addition, by 4 weeks postpartum, tissue will fully recover to virgin levels.

To make comparisons between tissues, the viscoelastic behavior was modeled with the quasi-linear viscoelastic (QLV) theory developed by Fung (1972). Specific forms of the generalized relaxation function and the elastic response were utilized along with an established approach to simultaneously fit the theory to experimental data from both ramping and relaxation portions of static stress-relaxation tests of virgin, pregnant, and post-partum cervical specimen [6,7]. The QLV constants A, B, C, τ_1, and τ_2 were determined and compared between groups.

METHODS

Cervical specimens were collected from virgin (n=5), mid-pregnant (14-15 days, n=4), and 4-week post-partum (n=4) Long-Evans rats. This study was performed with the approval of IACUC at the University of Pittsburgh (#0702224). The cervix was isolated from connective tissues, the uterine horns, and vaginal tissues by transecting proximally at the fusion of the uterine horns and distally at the dense fibers which transition into the vagina. The length, width, and thickness of the cervix were then measured using digital calipers (accuracy ±0.025). The average of three measurements was used to calculate cross-sectional area of the cervix, assuming rectangular geometry.

The whole cervix was centered in a custom container and submersed in a 0.9% saline solution at room temperature. The container was fixed to the base of a material testing machine (EnduraTec Elf 3220, Bose Corporation, Eden Prairie, MN), a custom plate was attached in series to a load cell (Honeywell, Columbus, Ohio), and the actuator of the material testing machine. Each specimen was loaded along the ventral-dorsal direction. Specimens were preloaded to 0.15 N, followed by preconditioning to 10% strain for 20 cycles at 1 Hz and

given 10 minutes to recover before stress-relaxation trials. Each specimen underwent stress-relaxation testing during which the specimen was compressed to 20% strain at a rate of 0.167 mm/sec and held for a period of 4 minutes followed by a recovery period of 30 minutes. Specimens were subjected to 3 trials of stress-relaxation tests.

The QLV theory developed by Professor Fung was utilized to characterize the viscoelastic properties of the virgin, pregnant, and postpartum cervix. The reduced relaxation function, $G(t)$, describes the soft tissues that are relatively insensitive to strain rate, and can be described as [4,5]

$$G(t) = \frac{1 + C\left[E_1\left(\frac{t}{\tau_2}\right) - E_1\left(\frac{t}{\tau_1}\right)\right]}{1 + C\ln\left(\frac{\tau_2}{\tau_1}\right)} \qquad (1)$$

where $E_1 = \int_y^\infty \frac{e^{-\xi}}{\xi} d\xi$ is the exponential integral, C, $\tau_1$and τ_2 are material constants. The dimensionless constant C defines the magnitude of viscous effects present and is related to the percentage of relaxation while the time constants τ_1 and τ_2 govern the initial and late relaxation, respectively. These constants are also related to the slope of the stress-relaxation curve defined by [6]:

$$\frac{dG(t)}{d(\ln(t))} = \frac{C}{1 + C\ln\left(\frac{\tau_2}{\tau_1}\right)} \qquad (2)$$

The instantaneous elastic response was approximated using the exponential function [4].

$$\sigma^e(\varepsilon) = A(e^{B\varepsilon} - 1) \qquad (3)$$

where A and B are material constants. The constant B and the product $A\times B$ are the rate of change of the slope of the stress–strain curve and the initial slope of the curve, respectively.

Rather than assuming instantaneous step change in strain, a method developed by Abramowitch et al. utilizes simultaneous curve-fitting of the QLV constitutive equations to the ramping and relaxation portions of the data and converges to a unique solution [5,6,7]. Therefore, the assumption of instantaneous deformation is no longer needed, allowing slow strain rates to be used in order to accurately approximate a ramp and hold strain history. Constants were estimated by entering the data from the third stress-relaxation trial for each specimen into an optimization routine developed in Mathematica (Wolfram Research, Inc. Champaign, IL) by Abramowitch et al. [5].

For statistical analysis a non-parametric Kruskal-Wallis test was performed on all viscoelastic constants with a Mann-Whitney post-hoc. The significance level was set at $p<0.05$ (SPSS, version 12.0.1, SPSS Inc, Chicago, IL)

RESULTS

Upon reaching a preloaded of 0.15 N the cervix quickly underwent relaxation. Therefore, no load was observed at the initiation of the preconditioning cycles. Also, the specimen experienced a reduction in maximal load during successive stress-relaxation trials. On average the maximal load decreased by 26%±22% (mean±SD) between the first and third trials, whereas the load decreased by no more than 10% between the second and third trials for all specimens. After three trials, the tissue response was repeatable. Thus, it was assumed that the reduction in maximal load, which was substantial (+60%) for some samples, was attributed to non-recoverable phenomena (Mullins Effect) possibly related to passive smooth muscle behavior, and was deemed outside of the scope of the present study. Under the current protocol, there were no obvious trends between groups in terms of the decrease in maximal loads across trials.

After the third trial, the peak stress was calculated for the three groups. Stress-relaxation curves were created by normalizing the stress to the peak stress, which occurred after a ramp time ranging from 2 to 4 seconds depending on tissue thickness (Fig.1). The total percentage of stress relaxation was 98.6%±1.54% (mean±SD) for the three groups with no observable differences between groups.

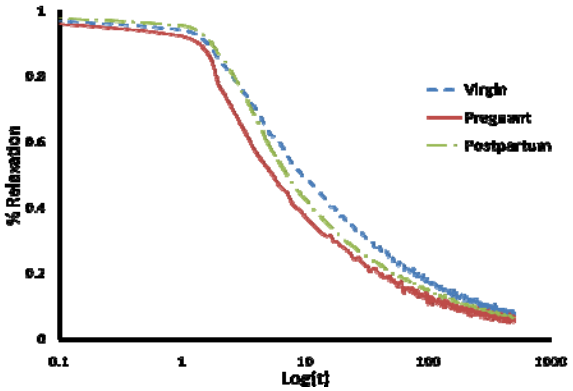

Figure 1.Representative stress-relaxation curves (normalized) for virgin (short dashed), pregnant (solid), and postpartum (long-short dashed) cervical tissues in response to unconfined compression.

TABLE 1. Median value of the constants describing the instantaneous elastic response of the virgin, pregnant, and postpartum Long-Evans rat cervix. P-values were attained using a Kruskal-Wallis test

	A(kPa)	B	A×B (kPa)
Virgin (n=5)	0.0466 (0.0079-0.412)	37.1 (29.8-42.1)	1.84 (0.32-5.87)
Pregnant (n=4)	0.0177 (0.0306-0.412)	36.16 (27.56-45.08)	5.05 (1.35-12.5)
Postpartum (n=4)	4.53 (0.593-15.4)	27.1 (20.6-32.7)	101 (19.4-329)
p	0.02	0.18	0.02

The constants describing the instantaneous elastic response for virgin, pregnant, and postpartum cervix are shown in Table 1. The constants A and $A\times B$ were significantly different between groups with the initial slope, $A\times B$, of the postpartum stress-strain curve measuring 98.2% and 95% greater than virgin and pregnant curves respectively (Fig 2.).

The three constants describing $G(t)$, for virgin, pregnant, and postpartum cervix are specified in Table 2. There were no statistical differences found between the three groups for the constants C, τ_1, and τ_2 ($p > 0.05$).

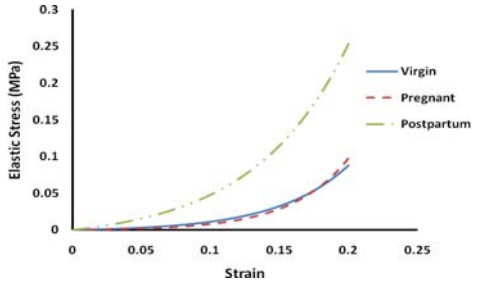

Figure 2.Representative curves for virgin (short dashed), pregnant (solid), and postpartum (long-short dashed) cervical tissues in response to unconfined compression.

TABLE 2. Median value of the constants describing the reduced relaxation function of the virgin, pregnant, and postpartum Long-Evans rat cervix. P-values were attained using a Kruskal-Wallis test

	C	τ_1	τ_2	dG/d(ln(t))
Virgin (n=5)	5.55 (2.77-7.18)	0.015 (0.0099-0.0165)	16.9 (14.9-28.1)	0.136 (0.0124-0.139)
Pregnant (n=4)	5.37 (4.88-5.98)	0.0127 (0.008-0.408)	9.98 (8.05-20.7)	0.149 (0.136-0.227)
Postpartum (n=4)	9.83 (3.81-16.8)	0.0262 (0.0149-0.033)	11.8 (4.15-17.8)	0.169 (.136-0.201)
p	0.789	0.359	0.337	0.122

DISCUSSION

In this study, the viscoelastic properties of the virgin, pregnant, and postpartum rat cervix were described using Fung's QLV theory allowing for differences to be observed between postpartum and virgin cervical tissue ($p<0.05$).

The data from this study refutes our hypothesis that the pregnant tissue will exhibit a less stiff and more viscous response than both virgin and postpartum specimens. The lack of softening in the pregnant group may be attributable to the fact that the pregnant animals were evaluated in mid-pregnancy, as the cervix is known to undergo the most dramatic changes near the time of delivery; we are now evaluating a late pregnant group. In addition, the loading direction utilized in this study (ventral to dorsal) was chosen because of the small size and irregular geometry of the rat cervix; however, this likely does not replicate in-vivo loading conditions, which may explain our findings. Although, since softening of the cervix can be detected clinically via palpation, it is anticipated that this protocol will detect changes in cervical mechanical behavior if they are present.

Another unanticipated finding was that, the postpartum tissue had the stiffest elastic response, as well as the greatest range between specimens for the constant *C* (9.83(3.81-16.8)). The change in post-partum tissue may reflect a pregnancy induced remodeling process that has not fully recovered back to virgin levels or, more likely, a healing response which results in a stiffer tissue. Future studies on the biochemical constituents will be used to clarify this result. A third interesting observation of this study was the sometimes dramatic reduction in the peak stress when exposing the cervix to consecutive stress-relaxation tests. Preliminary studies demonstrated that increasing the length of the recovery periods between tests had no effect on this result. Thus, we attributed this phenomenon to Mullins effect. Since the cervix contains a small percentage of smooth muscle and passive smooth muscle is known to exhibit Mullins effect [8], it may be that the smooth muscle component is causing this phenomenon. Future experimental protocols and modeling efforts will aim to better capture this phenomenon. Also, it has been reported that composition and structure of human cervix varies between the proximal and distal ends [2]. Therefore future studies will transect the rat cervix into a proximal and distal portion to create more uniform samples.

Finally, it should be noted that the constants obtained in this study have yet to be validated by predicting a second experiment with a different strain history. Nevertheless, based on comparisons to parameters obtained for other tissues and what we know compositionally about the cervix, the values that were obtained in this study are reasonable. Thus, the methodology utilized in this study appears to be appropriate to detect changes in the mechanical behavior of the cervix. In the future, we plan to more rigorously compare these results to human tissue to determine whether the rat can be used as a model to study the mechanics of the cervix.

ACKNOWLEDGMENTS

The authors acknowledge the financial support provided by NIH Grants-R01HD-045590 and K12HD-043441.

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I was born in Pittsburgh on February 27, 1987 and have lived here my entire life with my parents and older sister. I attended Mt. Lebanon High School where I played basketball and developed a love for biology and chemistry thanks to a series of amazing teachers in the sciences. I graduated from the University of Notre Dame in May 2009 with a BS in Biochemistry and a minor in Latino Studies. During my time there, I tutored chemistry and biochemistry and served as a resident assistant. I also volunteered at the Sto-Rox Family Health Center and the Birmingham Clinic during breaks from school.

In college, I further cultivated my passion for science working as a research assistant under biochemistry professor Dr. Jennifer Dubois. There I studied enzymes from two different organisms, *Klebsiella pneumoniae* and *Aspergillus fumigatus*, that are part of an iron sequestration pathway and could potentially serve as a novel antimicrobial target for these hard-to-treat pathogens. In the summer of 2008, I was fortunate enough to work with Professor Billy Day at the University of Pittsburgh studying the changes in nuclear matrix proteins between tamoxifen-sensitive cells and tamoxifen-resistant cells. In August of 2009, I will begin coursework at the University of Pittsburgh School of Medicine where I will continue research. I am excited to be working with Dr. Liang and Dr. Woo comparing wild type biological scaffolds and their Gal-deficient forms. Here I have already learned to work more independently than I have in the past, and I would like to thank Dr. Liang and Dr. Woo for giving me this opportunity.

Bioactivity of Gal-deficient ECM Extracts: Effects on the Proliferation of ACL Healing Cells

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INTRODUCTION

In recent years, biological scaffolds have become a prominent tool in functional tissue engineering. They are known to provide growth factors such as transformative growth factor-β1 (TGF-β1), fibroblast growth factor-2 (FGF-2), and vascular endothelial growth factor (VEGF) that assist in the healing process (ref. 1). Two scaffolds that are commonly used are porcine small intestinal submucosa (SIS) and urinary bladder matrix (UBM). Because these tissues come from a non-primate species, however, the Gal epitopes in the tissue could lead to immunorejection in the human host by reacting with the natural anti-Gal antibodies (ref. 2). A transgenic pig lacking the enzyme that attaches the Gal epitope to the tissue has been developed in hopes of limiting hyperacute rejections of xenotransplant organs (ref. 3). The goal of this study is to verify that extracts from Gal-deficient scaffolds have the same bioactivity as the wild type scaffolds. We hypothesized that the Gal-deficient scaffold extracts would have the same bioactivity as the wild type extracts, because the growth factor genes lie primarily on other chromosomes.

MATERIALS AND METHODS

Wild type and Gal-deficient SIS and UBM scaffolds were stored at -80°C. For growth factor extraction, tissues were removed from storage and ground with a mortar and pestle over liquid nitrogen. Tissues were lyophilized overnight, then re-suspended in 2 M NaCl and stirred at 4°C overnight. The samples were then centrifuged at 4°C for 30 minutes at 12000 g, and the supernatant was aspirated for storage at 4°C. The pellet was re-suspended in 50 mM Tris·HCl and 2 M urea with 10 mM protease inhibitors (EDTA, benzamidine, phenylmethylsulfonyl fluoride, and N-ethylmaleimide) and stirred overnight at 4°C. This digestion was centrifuged as before and repeated. The pellet was re-suspended in 50 mM Tris·HCl and 2 mg/ml collagenase and digested overnight at 4°C.

The digestion was centrifuged at 4°C and repeated at 37°C. Supernatants from every digestion were stored at 4°C. The NaCl digestion was combined with both urea digestions, and both collagenase digestions were combined. These samples were dialyzed in 0.1% SDS at 4°C for three days. All samples were lyophilized overnight and re-suspended in PBS to ~1 mg/ml. A biocinchoninic acid (BCA) assay (Thermo Scientific Pierce) was performed to determine the concentration.

The anterior cruciate ligament (ACL) of a goat was cut and allowed to heal for 12 weeks. The cells from the healing ACL tissue were then isolated and cultured in DMEM with 10% FBS and 1% penicillin/streptomycin. Media was changed every 3 days and cells were passaged every 6 days. After one passage, ~5,000 cells were plated in all but 12 control wells of three 96-well plates. These cells were allowed to attach to the plate surface over 48 hours.

Protein samples were diluted to 100, 10, 1, 0.1, and 0.01 µg/ml in DMEM with 0.5% FBS and 1% penicillin/streptomycin. The media from the cell culture plates was aspirated and replaced with 100 µl of protein sample. Three replicates were done for each concentration on each plate. The media was replaced every 24 hours with fresh protein sample. One plate was treated at each time point of 24, 48, and 72 hours. At the end of each time point, the protocol for EMD Biosciences BrdU Cell Proliferation Assay, HTS was followed. All samples were stored at 4°C after fixation, and the antibody sequences for all plates were run simultaneously. Data was collected using a Tecan Infinite M200 plate reader at 325 nm for excitation and 420 nm for emission.

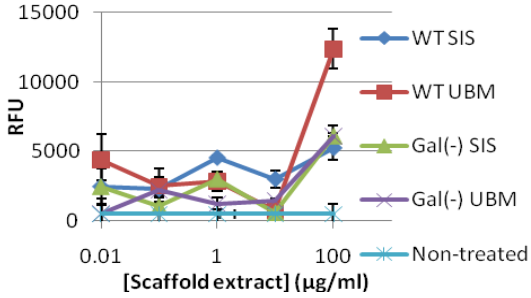

Figure 1. Cell proliferation at different concentrations of growth factor from four ECM bioscaffolds

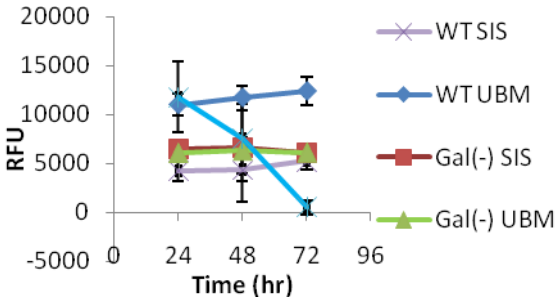

Figure 2. Cell proliferation over time at 100 µg/ml of bioscaffold extract as compared to non-treated cells.

RESULTS

After subtracting out the blank, the relative fluorescence units (RFUs) of the 100-µg/ml treatment at 72 hours were significantly different from untreated cells (Fig. 1). When the data from the 100-µg/ml treatments were plotted over time (Fig. 2), the proliferation of non-treated cells decreased significantly over time while the treated cells maintained a relatively steady proliferation level. The wild type UBM extract had a higher level of proliferation than other three scaffold types.

DISCUSSION

The extracts of all four scaffolds from the wild type and Gal-deficient pigs were determined to be bioactive. We had hypothesized that the Gal-deficient scaffold extracts would have the same bioactivity as the wild type extracts, because the growth factor genes lie primarily on other chromosomes. Figure 2 shows that extracts from all four scaffold types sustain cell proliferation, while proliferation of non-treated cells slows significantly over the 72-hour period. All four scaffold extracts are therefore bioactive, including the Gal-deficient ones.

The wild type UBM extract appeared to yield a higher proliferation than the other bioscaffolds. This is likely due to an elevated level of TGF-β1 found in the WT UBM sample as demonstrated by Western blot (Fig. 3). Extracts from more scaffolds must be performed to determine why this could be. Based on the consistency between the wild type and Gal-deficient SIS tissues, we anticipate that more replicates will demonstrate a consistency between Gal-deficient and wild type UBM extracts as well.

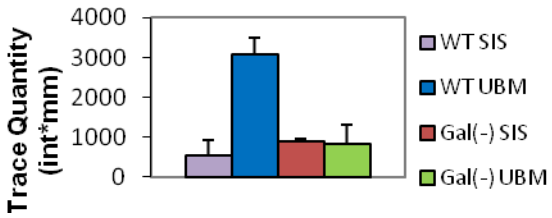

Figure 3. Levels of TGF-β1 in extracts of each scaffold type. The TGF-β1was significantly greater in the wild type UBM sample.

It will also be necessary to ensure that all cells are proliferating at the same rate at t=0. This will be done by performing a BrdU assay at t=0 in addition to the three previously measured time points. It will also be interesting to see how the scaffold extracts affect gene expression. This can be done by performing RT-PCR on the treated and non-treated cells to determine whether each type of scaffold affects expression differently.

ACKNOWLEDGEMENTS

I would like to thank Dr. Rui Liang for all of her guidance in the lab and her patience as she helped me to revise my work. I would also like to thank my faculty mentor, Dr. Savio L-Y. Woo, for the chance to work in his laboratory this summer and the Pittsburgh Tissue Engineering Initiative for giving me this opportunity.

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Tissue Mechanics
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I was born the 28th day of July in 1986 in a suburb 25 miles east of Pittsburgh, called Jeannette. I grew up alongside my older sister, Crystal, and under the watchful eyes of my wonderful parents Tom and Kathy. I attended Hempfield Area High School in Greensburg, PA where I was a member of many clubs including National Honor Society, French Honor Society, and the marching band.

After high school, I attended Saint Vincent College in Latrobe to major in Math/Engineering. During my three years there I became a member of Alpha Lambda Delta, Alpha Phi Omega, Orientation Committee, as well as many other small clubs and organizations. I transferred to the University of Pittsburgh in the fall of 2007 to complete my engineering degree and major in Bioengineering. I am an active member of BMES and the current Vice-President of Tau Beta Pi engineering honor society.

This is my second summer in the MSRC and I have thoroughly enjoyed both experiences. I will take so much knowledge with me that I have gained in my time here; not only in the subjects of bioengineering and biomechanics, but also knowledge for life in general. I would like to thank Andrew Feola and Steve Abramowitch for taking a chance on me, and Dr. Woo for all his wisdom and the effort that keeps the MSRC running. I would also like to thank my fellow summer students for all of their help and the laughs along the way.

CHARACTERIZING THE ANISOTROPIC BEHAVIOR OF THE RAT VAGINA

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INTRODUCTION

All biological materials contain oriented fibrous structures and thus display mechanical anisotropy when loaded in more than one direction[1]. Anisotropy is a term that describes a material that has different mechanical responses along different axes. Some biological materials that exhibit anisotropy include the urinary bladder[2] and various types of blood vessels[3]. These organs depend on the tissue to distend greatly in the circumferential tissue axis. However, in both cases, the longitudinal tissue axis does not need to expand quite as much. Therefore, the urinary bladder and vasculature require a structure that allows the circumferential axis to distend more than the longitudinal in order to maintain normal physiologic function.

The vagina is another perfect example of an anisotropic material due to the need for extreme distensibility in the circumferential tissue axis, especially during the second stage of labor to avoid maternal birth injury[4]. Previously, in our center, numerous uniaxial tests have been done on the vagina[5]. However, due to anisotropy and collagen fiber rotation, the uniaxial test results may not be indicative of the true mechanical properties of the tissue. Biaxial mechanical testing is advantageous in that it allows one to simultaneously load two axes of a tissue and determine the extent of anisotropy that the tissue exhibits. Due to clamping on all tissue sides, biaxial testing also prohibits extensive collagen fiber rotation and allows the true biomechanical properties to be measured. Furthermore, with the assumption that all biological materials are incompressible[1], in the future we will be able to model the three-dimensional tissue behavior using the two-dimensional biaxial mechanical data.

For this study, we will use a rodent model to determine the anisotropic mechanical response of a virgin vagina and more specifically to quantify the biaxial mechanical properties of the rat vagina. We will determine the maximum tangent modulus (MTM), areal strain, and anisotropic index (AI) of virgin vaginal tissue under equibiaxial stress conditions.

METHODS

8 virgin Long Evans rats were used for this study. The protocol was performed in accordance with Institutional Animal Care and Use Committee guidelines. After sacrifice, the rats were dissected down and the reproductive tissues were isolated and frozen in saline soaked gauze at -20°C.

On the day of testing the specimen was thawed and the vagina was isolated and cut across the proximal edge just below the cervix and along the distal end just above the vaginal opening. The vagina was then cut open along the urethra to produce a planar-rectangular specimen. Width and length were measured using digital calipers while tissue thickness was measured across three areas of the vagina using a non-contact laser micrometer[6]. A total of 16 hooks were placed around the tissue (four on each side). The tissue was then mounted onto the Bose Electroforce LM1 Testbench testing system using custom-made clamps that distribute load evenly across each side of the specimen and allow for freely moving edges. Five graphite strain tracking markers were placed centrally on the specimen and strain was calculated using a strain camera. The specimen was completely immersed in a room-temperature 0.9% saline solution for the duration of testing.

Before each test, the specimens were preloaded to 0.1 N on each independent axis. The specimens were then preconditioned for 10 cycles to the maximum stress of 100 kPa on each axis over an interval of 15 seconds. All test protocols maintained a constant axial stress ratio in which peak stresses were controlled to be 15, 30, 50, 75 and 100 kPa in each respective test[2]. The various stress levels were carried out to ensure repeatability of the vagina sample after preconditioning.

Areal strain and AI values are expressed as mean ± standard deviation while MTM and maximum strain are expressed as median (25-75 quartiles). A non-parametric Mann-Whitney test was performed on MTM and maximum strain between the longitudinal and circumferential directions, and a one-sample t-test was performed to determine if the AI was significantly different from zero (zero = isotropic). All statistical tests had a significance level set to $p<0.05$.

RESULTS

All tests resulted in a non-linear stress versus strain relationship with some degree of mechanical anisotropy between the tissue axes. Figure 1 displays a representative curve of the biaxial mechanical response of rat vaginal tissue to a maximal stress of 100 kPa. This figure demonstrates a longer toe region and therefore a larger maximum strain in the circumferential tissue axis.

The MTM was determined for each individual specimen along each tissue axis using the slope of the upper-linear region of the stress strain relationship curve. Specific values for the MTM can be found in Table 1. On average, the longitudinal tissue axis had a higher MTM than the circumferential axis ($p = 0.015$). The larger MTM for the longitudinal direction indicates some degree of anisotropic behavior between the tissue axes.

The maximum strain in each direction was recorded and can be found in Table 1. As expected, the circumferential direction, on average, strained much more than the longitudinal axis for all of the virgin specimens ($p = 0.005$).

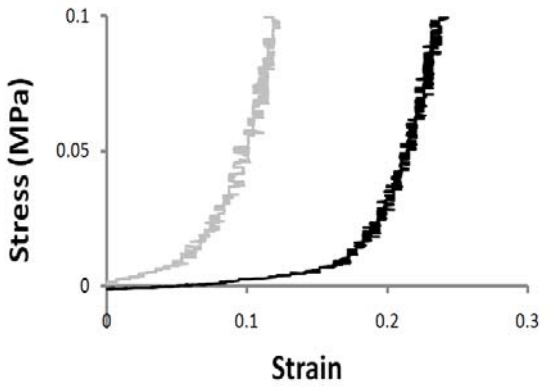

Figure 1. Representative Stress/Strain curve showing longitudinal axis in light gray and circumferential axis in black

Table 1: Biaxial Mechanical Properties of Vaginal Tissue represented as median (interquartile range)

MTM Long (MPa)	MTM Circ (MPa)	Max Strain Long	Max Strain Circ
2.52 (2.14— 3.19)	1.88 (1.85—2.12)	0.041 (-0.015—0.088)	0.156 (0.107—0.187)

Areal strain is a measure of the fractional difference in specimen area between the loaded and unloaded states[2]. It is calculated using the maximum strain in each tissue axis for a given specimen. These results show that the areal strain for the virgin specimens is roughly 0.21 ± 0.07 (Table 2).

The anisotropic index is a measure of the degree of anisotropy between the mechanical responses of different tissue axes[2]. It is also calculated using the maximum strain in each tissue axis for a given specimen. The anisotropic index for the virgin specimens was found to be about 1.20 ± 0.95 (Table 2). The t-tests revealed that the anisotropic index was significantly greater than zero (p=0.009). Therefore, the virgin rat specimens conclusively display anisotropy.

Table 2: Areal Strain and Anisotropic Index represented as mean ± standard deviation

Areal Strain	AI
0.21 ± 0.07	1.20 ± 0.95

DISCUSSION

In this study, we used biaxial mechanical testing to an equibiaxial stress ratio to determine the anisotropic behavior of the rat vagina. The findings of this study show that there is a non-linear stress strain relationship in both the longitudinal and circumferential axes in virgin vaginal tissue. Additionally, we have determined that the vaginal tissue is indeed anisotropic with differences in both the MTM and maximum strain between the tissue axes as well as an anisotropic index greater than zero.

We expected to see a greater difference in MTM between the tissue axes to correlate to the anisotropic nature of the tissue, as suggested by the anisotropic index. However, the anisotropic index is based solely off of the maximum strain achieved in each tissue axis. Therefore, in this case, the anisotropic index may merely reflect the longer toe region in the stress strain relationship for the circumferential tissue axis as illustrated by Figure 1 and not a smaller MTM as originally expected. We believe that this behavior can be attributed to collagen fiber rotation into the circumferential tissue axis to allow for a greater distensibility during labor. Similarly, extensive collagen crimping in the circumferential axis could also create a longer toe region in a common stress strain curve. Both of these instances may show that virgin rat vaginal tissue may already contain some mechanical properties that prepare it for labor. In testing the pregnant specimens, we hope to see that these properties are exaggerated even further.

Previously, in our lab, uniaxial testing has been done using the longitudinal axis of rat vaginal tissue. The results from these tests show that the MTM of the virgin specimens in the longitudinal axis is 25.1 ± 5.1 MPa. This is about 22 MPa greater than the MTM found in this biaxial study for the longitudinal tissue axis. We can attribute this discrepancy to collagen fiber rotation into the longitudinal tissue axis because in uniaxial testing the circumferential collagen fibers would not be constrained and therefore could freely rotate. More collagen fibers in the longitudinal direction would cause a greater stiffness response and therefore higher MTM in the uniaxial study.

This biaxial study produced the first results obtained from the biaxial testing protocol in our lab. Therefore, we wanted to validate that the values we were finding were reasonable. Biaxial literature values for the urinary bladder wall show that it has an anisotropic index of about 2.0 and an areal strain around 0.22[2]. This is comparable to the values found in this study for rat vaginal tissue. Therefore, the values found in this study are reasonable and on about the right order of magnitude.

Future work remaining for this study includes completing the biaxial testing on the pregnant specimens that have been recently obtained. Furthermore, we will be looking at and comparing the biaxial mechanical properties including the MTM, maximum strain, AI, and areal strain between the virgin and pregnant specimens. We expect that the MTM will decrease for both tissue axes, the areal strain will increase, and the anisotropic index will increase in the pregnant compared to the virgin specimens.

ACKNOWLEDGMENTS

I would like to thank Andrew Feola and Steve Abramowitch for all they have taught me over the last year and a half. I would also like to say thanks for all of the time Steve, Andrew and the other faculty and grad students spend on me and the other summer students. We are forever grateful that you choose to share your knowledge with us. I would also like to thank Dr. Woo and the rest of the MSRC for making the lab a great place to work and learn.

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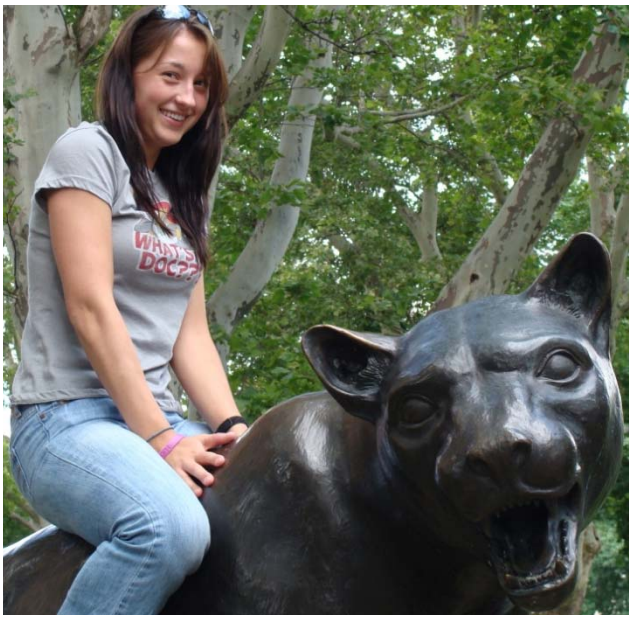

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Shoulder Group
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Born and raised in small town Wisconsin, living in the Pittsburgh area for the summer was my first out of state adventure lasting over a month. I grew up being the oldest of two girls and was a kid with an insatiable curiosity for science. I knew I wanted to become a doctor at the age of 9 when my grandmother died of lung cancer. I continued to pursue my interest in biology through high school and into college; this fall marks my fourth and final year as a Biological Science major at University of Wisconsin Parkside. Though I've made some wonderful friends and had great experiences there, nothing beats my trip to Nicaragua where I spent two weeks working in medical clinics, hiking to waterfalls, kayaking a lake, swimming in natural springs and even climbed to the top of Volcano Maderas.

Working at the MSRC has given me the opportunity to explore the ever changing field of bioengineering, something I had never heard much about before. My peers in the shoulder group, my advisor Carrie and my PI Dr. Debski have all been very patient in teaching me the fundamental concepts of biomechanics. I now have a more complete knowledge of the glenohumeral joint, and the properties of its capsule. I know more about ligaments and collagen fiber orientation and alignment than I ever learned in my college Anatomy course and I have my mentors at the MSRC to thank for that. I survived a Stanley Cup summer in Pittsburgh!

COLLAGEN FIBER ORIENTATION IN THE GLENOHUMERAL CAPSULE DURING UNIAXIAL EXTENSION

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INTRODUCTION

The shoulder is the most dislocated joint in the body. About 5.6 million Americans (2%) dislocate their shoulder [1], and about 90% of these individuals will need surgical repair to prevent future dislocations [2]. The glenohumeral capsule is a continuous sheet of ligamentous tissue [3] that connects the scapula and humerus. This capsule is typically divided into discrete anatomic regions. The anteroinferior region of the capsule consists of the anterior band of the inferior glenohumeral ligament (AB-IGHL), the posterior band (PB-IGHL), and the axillary pouch. The IGHL is the region most commonly injured during dislocation as more than 75% of dislocations occur in the anterior direction [4].

Biological soft tissues such as ligaments and tendons are composed primarily of collagen, the main load bearing tissue in the body. Collagen makes up about 80% of dry weight of the anteroinferior capsule [5]. The axillary pouch when unloaded has been shown to have regions of aligned fibers in an overall randomized fiber distribution. [3] The effect of loading on the collagen fiber orientation of some biologic tissues has been examined. Both the supraspinatus tendon and SIS showed an increase in fiber alignment in the direction of loading [6] [7]. Therefore, it was hypothesized that the collagen fibers of the axillary pouch will become more aligned with loading. To test this hypothesis, a protocol was developed and collagen fiber alignment of the axillary pouch was quantified while the tissue was loaded to failure.

METHODS

In order to develop an experimental protocol to quantify the collagen fiber alignment, preliminary experiments were completed to assess the feasibility of each step. The small angle light scattering (SALS) device can accurately determine the collagen fiber alignment of tissue samples 500 μm thick or less. [8] Therefore, tissues were originally sliced at 100 μm, 250 μm, 300 μm and 400 μm sections. After attempting to place each of these tissue sections either in the soft tissue clamps or suture them into place, it was learned that thicker tissue slices are more appropriate for this study, as thinner slices tore before being secured into place. Training was also required to properly use the cryostat for sectioning the tissues. The stretching device attached to SALS device then had to be modified to carry out uniaxial extension of each tissue sample. The device was intended for biaxial tissue testing using sutures to hold the tissue. [7] Inserting these sutures was tedious and time-consuming, and often the tissue would tear or fold back on itself, creating an area too thick to scan with the SALS device. The horizontally oriented pulleys were removed from the stretching system, and the vertical pulleys were replaced with soft tissue clamps. This modification allowed for the tissue to be secured quickly and remain as flat as possible by avoiding self-folding. A rectangular tissue sample (30 mm x 15 mm) was extracted from the axillary pouch of a fresh-frozen 66 year old male cadaveric shoulder. Samples were sliced to a thickness of 400 μm on the cryostat and were placed in custom designed soft tissue clamps. The tissue was then preloaded to 0.2 N (Instron) and clamp-to-clamp distance was recorded. The clamps were then integrated into the stretching device [7] and submerged in saline. Collagen fiber alignment was determined using a SALS device. [8] The samples were elongated at 8% increments based on the clamp-to-clamp distance and the collagen fiber alignment was determined following each elongation. This procedure continued until visible rupture of the tissue was evident. The orientation index was calculated after each elongation [8] and a normalized percentage (NOI) was computed such that higher values indicated increased fiber alignment. The mean preferred fiber direction (PFD) was also calculated [8] at each elongation.

RESULTS

As the tissue is elongated from its preloaded state to 8%, 16%, and 24% elongation, the NOI percentage values increase by more than 25%. The standard deviations for these values decrease by nearly half as the tissue is elongated from its preload to 24% elongation, indicating a reduction in variation of the collagen fibers and an increase in their alignment. The PFD values decrease by about 20% from the preloaded state to 24% elongation. The standard deviations for these values are reduced by more than half from the preloaded state to 24% elongation, also indicating an increase in fiber alignment in the direction of loading.

The scale at right in **Figure 1** coordinates colors with NOI percentages, such that blue and green reflect areas of minimal fiber alignment and pink and red areas reflect highly aligned regions. Yellow regions reflect moderate collagen fiber alignment. The tissue in the preloaded state consists of mostly blue and green areas, whereas at 8% elongation the tissue is mainly green with fewer, smaller blue regions. At 16% the tissue is mostly yellow in color with fewer green and blue regions. At 24% elongation, the tissue contains a large red/pink region and nearly no blue areas. This suggests that as the tissue is elongated the collagen fibers become increasingly more aligned.

DISCUSSION

The changes in collagen fiber alignment of the axillary pouch of the inferior glenohumeral ligament with uniaxial loading were examined in this study. As the tissue was elongated from a preloaded state to failure, collagen fiber alignment increased in the direction of loading. This is reflected by the increase in the NOI values, measurements that signify the mean collagen fiber distribution. An increase in the NOI corresponds to an increase in collagen fiber alignment. The standard deviations for these values continually decreased, also indicating an increase in fiber alignment. Also, PFD values decrease, which means the mean collagen fiber angle decreases. As mean fiber rotation approaches 0°, this signifies that fibers are continuously aligning more in the direction of vertical loading. These findings compare well with previous studies that found collagen fiber align with the direction of loading in the supraspinatus tendon. [6]. Lake et. al found that fiber realignment occurred only in the stiffer Anterior-Bursal side of the tissue sample as well as the Anterior-joint side of the tissue sample [6], rather than other less stiff tissue regions. When Gilbert et al uniaxially extended SIS specimens, they found that the mean collagen fiber distribution rotated toward the distribution of stretch with progressive increase in fiber alignment [7]. These findings are identical to those found in this study.

These findings suggest that collagen fibers align when loaded and could indicate a permanent change in the collagen fiber make-up of glenohumeral tissue. If collagen fibers remain aligned following injury to the tissue, this may contribute to dislocations in directions other than that of the alignment of the fibers. However, there were several limitations posed in the process of this experiment. In order to develop the experimental protocol, only one tissue sample was examined. This sample had previously been used for a different experiment not related to fiber quantification studies. Because no load cell was present in the stretching device, forces were not quantified during loading. Percent elongation for the tissue samples was also measured with a ruler on the outside of the saline bath casing. Multiple freeze-thaw cycles of the tissue sample and transport in clamps from the materials testing machine to the stretching device did not, however, affect the fiber alignment.

In the future, this study should be repeated in the same manner until tissue sub-failure and more samples should be used from multiple cadaveric shoulders. A load cell should also be incorporated into the custom stretching device so loads are quantifiable during loading. Data from this and future studies may be used to create a constitutive model to predict collagen fiber alignment patterns prior to and following injury. Knowing the microstructural makeup of the tissue is imperative to understanding its function and investigating proper surgical repair techniques to prevent further glenohumeral dislocations from occurring.

Table 1. Normalized orientation index (NOI) and preferred fiber direction (PFD) values at each tissue elongation.

State	NOI (%)	PFD (°)
preload	37.2 ± 11.2	12 ± 23.3
8%	47.6 ± 8.7	11.6 ± 22
16%	48.4 ± 6.9	11.4 ± 15.6
24%	50 ± 6.2	9.5 ± 11.4

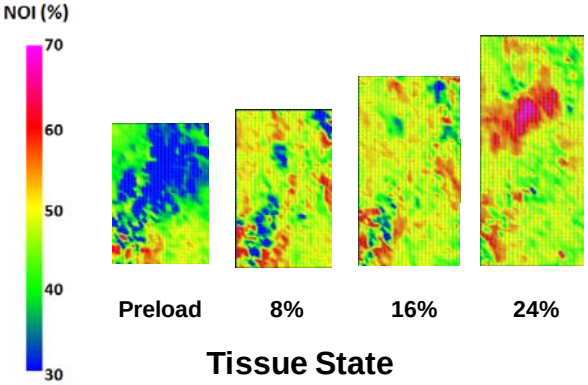

Figure 1. Tissue state with normalized orientation index (NOI) depicted by color scale at the left.

ACKNOWLEDGEMENTS

This experimental project would not have been possible without the patience, assistance and lessons in biomechanics from my mentor, Carrie Voycheck, and advisor, Dr. Richard Debski. In addition, I'd like to thank Dr. Abramowitch and Dr. Woo, who supported this project along with the rest of the MSRC. A sincere appreciation of the support from Pittsburgh Tissue Engineering Initiative is also noteworthy. This was both an educational and rewarding experience as I pioneered both into the fields of scientific research and bioengineering.

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I was born on August 23rd, 1989. I grew up playing baseball on the south side of Chicago. From tee ball to travel ball to high school, I played a wide array of positions, having to stop each time due to injury. I caught until my knees hurt constantly. Then I pitched until I couldn't throw without my elbow hurting. I ended up in the outfield, but I just wasn't as good out there. Meanwhile, I was attending school, playing basketball, baseball and soccer in grade school and opting for scholastic bowl in high school.

I attend Carnegie Mellon University and will be a Junior in the fall. I'm a mechanical and biomedical engineering double major, in the biomechanics track. My sports-related injury history sparked my interest in orthopedics and biomechanics. So far the coursework and this internship have been nothing but reaffirming of that interest.

The MSRC has been great for me. It was a great introduction to research and work in the field of biomechanics. The MSRC is a great forum for learning about the research process and all the work that goes into the preparation and presentation of experiments, as well as the mechanics of the experiments themselves. I have to thank Drs. Woo, Debski and Abramowitch for creating this forum, as well as Hilda Diamond for making the connection between CMU and the valuable teaching tool that is the MSRC.

STRUCTURAL PROPERTIES OF THE MEDIAL PATELLOFEMORAL LIGAMENT: WHY IN VITRO TESTS MUST REPLICATE IN-SITU CONDITIONS

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INTRODUCTION

Lateral patellar dislocation occurs frequently in physically active patients, particularly in the 10-17 age group. The medial patellofemoral ligament (MPFL) has been identified as the most important soft tissue restraint against lateral patellar instability and movement.[1] 94%-100% of lateral patellar subluxations are due to or result in the failure of the MPFL.[2] The best currently available treatment option is MPFL reconstruction, but there is no consensus in the literature about which technique, particularly which graft tissue) is the best technique. For this reason, we want to determine the structural properties of the bone-MPFL-bone complex (BMBC) to compare to potential graft tissues.

A previous study was done at the MSRC on this same topic, but the failure modes they reported were different from clinical observations, with the majority of the failures occurring in the midsubstance and at the patellar insertion, while clinically it is observed that the overwhelming majority of injuries occur at the femoral origin. For this reason, our study looks to recreate this experiment with a method more representative of physiological conditions. Thus the research question is as follows: Does replication of the physiological angle between the patella and the MPFL cause the BMBC to show failure locations more consistent with clinical data? Furthermore, does this alteration cause a change in the observed structural properties, such as ultimate load, ultimate elongation or stiffness of the BMBC as compared to the previous study?

We hypothesize that the failure locations will be more consistent with clinical observations than the previous study, with the majority of the failures being at the femoral attachment. This should happen due to a stress distribution over more tissue near the patellar attachment of the MPFL. With a stronger patellar attachment, it leaves the low cross sectional area of the femoral attachment to bear the highest stress. We also expect a higher ultimate load and stiffness and lower ultimate strain, consistent with the literature.[3]

MATERIALS AND METHODS

A jig was constructed that allows us to clamp the patella parallel relative to the MPFL, and that allows us to adjust the angle of the patella in increments of 10 degrees to recreate the physiological angle. In order to accomplish this, holes are drilled around the outside of two semi-circular plates, through which we will put through to lock the jig in place. On the inside of the circles, two slots are cut to allow the adjustment of two inner plates, which will sandwich and house the bolts that hold the patella in place. The slots are slightly offset from the center of the mechanism to keep the insertion site in the line of action of the Instron machine regardless of rotation.

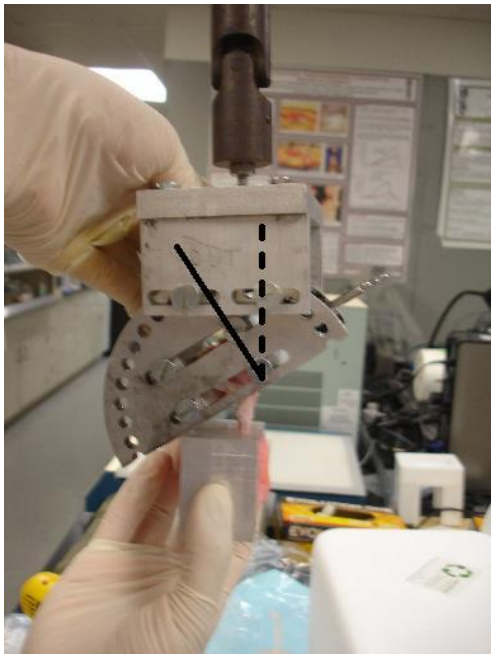

Figure 1: Angle (solid) of the patella as compared to the straight (dashed) orientation. The apparatus is loaded as shown for the angled tests.

The jig will then be set up on the Instron machine and adjusted so that the patella is oriented at a 150^{o} angle relative to the MPFL. The femoral bone block is potted at a ~37^{o} angle relative to vertical to ensure physiological orientation of the femoral origin of the MPFL. The jig is placed in a bath of saline solution heated to 37^{o} C. It then rests for 30 minutes. The specimen is preloaded to 2N, and the gauge length is reset. The ligament is then preconditioned with 10 cycles from 0mm to 2mm elongation. After preconditioning, the tension is lowered to 2N. When the ligament is at 2N, the ligament is loaded to failure. All tests are performed at 10mm/min. The load-elongation curve produced is then used for data analysis. 7 porcine knees will be tested in the angled orientation to match the seven porcine knees tested in the previous study in the straight orientation. In the extension of this study, more porcine knees will be tested in both orientations and the study will eventually be extended to human cadaveric specimens.

RESULTS

It was observed that in the angled orientation, five of the seven BMBCs failed in the femoral attachment, while only two failed in the midsubstance. It is important to note that the two midsubstance failures occurred near the femoral attachment, consistent with clinical observations. Meanwhile, the straight tests yielded two femoral failures, three midsubstance failures, and two patellar failures. A chi-square test was performed to compare failure modes in the

two test orientations, and the difference was found to be statistically significant (α=0.05, ν=2). The load-elongation curves were analyzed and there was no statistically significant difference between any of the observed structural properties between the old (straight) BMBCs and the new (angled) BMBCs (α=0.05).

	Straight			Angled		
	Ult. Load	Ult. Elong.	Stiffness	Ult. Load	Ult. Elong.	Stiffness
Average	347.1	9.2	63.5	384.4	10.8	59.3
Std. Dev	55.8	1.7	18.6	28.3	2.5	9.0
% Std. Dev	16.1	18.5	29.3	7.4	23.5	15.2
Failure Modes	Femur	Mid	Patella	Femur	Mid	Patella
	2	3	2	5	2	0

Table 1: Summary of Data

DISCUSSION

We believe the results of this study were inconclusive in part due to the low sample size of the study. We also feel there is a need to repeat the straight tests with the new clamp to ensure the uniformity of the testing conditions. By testing in the straight configuration in the new clamp, we hope to remove any confounding variables between tests. One observation of interest was the fact that the failure mode changed, but the structural properties did not change significantly. We suspect that this is due to either variation between experiments (different knees, different clamp, etc.) or low sample size. While the power analysis resulting from the preliminary tests showed that only five and six knees were needed to show statistical significance in the differences in ultimate elongation and stiffness, respectively, the differences in means and assume standard deviations were not close enough to those of the actual tests to reasonably determine needed sample size. Further analysis on more representative preliminary data will be required for the extension of the porcine testing.

The clamp was successful in its aim to allow straight setup for both straight testing and angled testing. It was sufficiently adjustable and minimized moments be centralizing the patellar attachment of the MPFL, thus conserving the line of action of the Instron. One concern for the future of the clamp is the uneven weight distribution caused by the semi-circular plates not being centered on the line of action. A counterweight may be considered in future tests to account for this difference.

CONCLUSION

The change in orientation during testing was able to cause changes in the failure mode of the BMBCs, but it was not able to show a change in the observed structural properties. Further tests are required to validate these results. The clamp was a success overall though, and barring minor adjustments, it should prove useful in these future tests.

ACKNOWLEDGEMENTS

I would like to thank my mentor, Kwang Kim as well as Dr. Hsu and Dr. Woo. I would also like to thank Hilda Diamond for connecting me with the MSRC.

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I was born in Kingston, PA on August 9th, 1990. I then moved to a small town called Dallas, PA where I lived with my parents and younger sister. Growing up I spent most of my time playing outdoors with the other kids in my neighborhood. I attended Dallas High School where I played varsity soccer and field hockey. I graduated in 2008 and decided to attend the University of Pittsburgh in the field of bioengineering. I am unsure of what I want to do after I graduate, but I know I want to continue my education, whether it be medical school, dental school, or graduate school.

While at Pitt, I enjoy many different activities. I am in the Phi Eta Sigma honor society and the National Society of Collegiate Scholars. I have participated in Student Leaders in International Medicine and I am a member of the Society for Women Engineers. In my free time I enjoy fencing with the Pittsburgh Fencing Association and exploring Pittsburgh with my friends. I hope to get my BS in bioengineering with a concentration in cellular and medical product engineering.

My experience at the MSRC has been very rewarding. I have gained an immense amount of knowledge dealing with bioengineering research that will help to further my career in medicine or engineering. I would like to thank Dr. Abramowitch, Dr. Moalli, Suzan Stein, and all of the MSRC staff for making my research experience very enjoyable and educational.

THE EFFECT OF A SOY-BASED DIET ON THE COLLAGEN ALIGNMENT OF THE UTEROSACRAL LIGAMENT

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INTRODUCTION

Pelvic organ prolapse is a condition in women in which one or more of the pelvic organs shift downwards, resulting in immense pressure on the vaginal canal. This happens when the pelvic floor muscles and/or connective tissue become weak or damaged and can no longer support the pelvic organs [1].

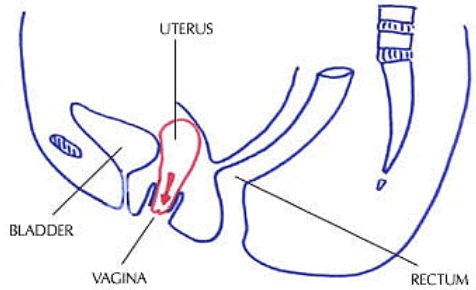

FIGURE 1
Example of uterine prolapse

Pelvic organ prolapse occurs in about 50% of women over the age of fifty. This is not considered a life threatening disease; however, it causes much discomfort in women and the costs of surgeries each year to correct this exceeds one billion dollars [1]. The type of pelvic organ prolapse that is relative in this study is uterine prolapse, which is where the uterus enters the vaginal canal, as seen in Figure 1 [2].

The main cause of pelvic organ prolapse is childbirth. This severely weakens the muscles and connective tissue in the pelvic floor. Pelvic prolapse can also be caused or worsened by obesity [3]. Because so many women suffer from prolapse there is much research on treating and preventing this condition. There are many theories on what factors will decrease the risks of developing prolapse, one of them focusing on diets. This particular study will investigate the effect that a soy-based diet has on the uterosacral ligament (USL), one of the main ligaments supporting the uterus.

Why Study Soy?

Soy protein is very low in fat, cholesterol and lactose. The benefits from soy primarily come from its isoflavone content. This is a type of antioxidant that helps combat cell damage and can also have an anabolic effect by aiding in repairing damaged muscle tissue. [4]. It is also theorized that the impact soy has on the body is affected by hormones. After menopause, the amount of estrogen in the body decreases, while the number of estrogen receptors increase. The isoflavones can act like estrogen and bind to the estrogen receptor sites [4]. This decreases and eases the effects of menopause, such as the weakening of muscles and connective tissue. It is hypothesized that incorporating soy into the diet will increase the strength and durability of the uterosacral ligament, and therefore help prevent pelvic organ prolapse. This hypothesis was partially derived from the fact that Asian women have been shown to have significantly less pelvic organ mobility than Caucasian women [5]. One of the main differences in diets between Asian and Caucasian women is the amount of soy protein that is consumed. This study will determine whether or not soy plays a role in the collagen alignment of the uterosacral ligament, and whether the presence or absence of hormones changes when soy is most beneficial (pre or post menopausal).

METHODS

This study involves Female Cynomolgus macaques, which have similar gestation cycles and menstruation cycles to humans. There were 30 macaques used during this study. They were divided into two groups: one was given the control protein and the other was given the soy protein. The control protein was casein. Casein is the protein found in milk that is common in most diets. The reason casein is the control for this experiment is that it is isoflavone free. The diets were otherwise kept the same. After about 30 months of this treatment, the primates all underwent an oophorectomy, which is a surgical removal of the ovaries. This induces menopause because there are no longer any hormones being produced.

After the oophorectomy, the groups were each split into two more groups and given either the soy or the casein for another 30 months, creating four groups overall. This not only allows for the testing of whether soy has an effect of the strength of the uterosacral ligament, but also if hormones can effect when the soy has an effect (pre or post menopausal).

The uterosacral ligaments are then embedded into blocks and sectioned on to slides. Slide thickness is 6-8 micrometers. About eight slides were cut from each uterosacral ligament. Three of those slides underwent a trichrome stain. Although this gives no data or quantitative numbers from the tissue, when observed by a high powered microscope, it shows what the ligament is made of and what type of tissue will be tested.

Two of the eight slides were selected for the picrosirius red stain. The picrosirius red stain uses chemicals, mainly picric acid and the solid compound Sirius red, to stain the collagen in the tissue a reddish color. This leads to the quantitative data that was gathered during this experiment. From the collagen stain, a collagen alignment ratio was

obtained. The picro-sirius red stain, when observed under a polarized light microscope, stained the aligned collagen red, while the unaligned collagen was stained green. The computer program was then used to separate the red and the green collagen. An example of the separated red collagen can be seen in Figure 2.

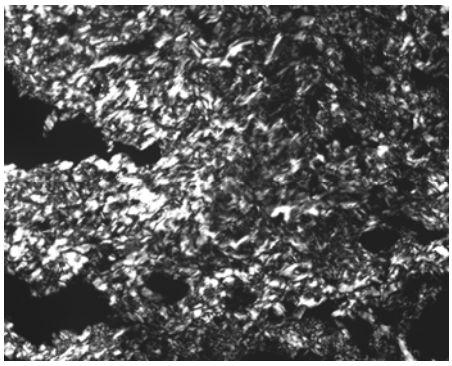

FIGURE 2
Example of red collagen separated from a slide of tissue using the microscope

Using a computer, the amount aligned collagen was determined and compared to the amount of unaligned collagen, and a collagen ratio was calculated. This was done by setting a threshold value on the computer program that determined the amount of significant collagen in each picture. The amount of pixels in the selected collagen is then obtained by the program. These numbers mean noting alone, but an accurately representative ratio can be calculated by dividing the amount of unaligned (green) collagen into the amount of aligned (red) collagen. Better aligned collagen indicates stronger tissue. Therefore, groups with a higher collagen ratio are stronger and healthier than groups with a lower ratio.

RESULTS

Five representative pictures were taken using the polarized light microscope from each uterosacral ligament. Thus, there were five collagen alignment ratios obtained from each sample. The ratios were then averaged to get one representative ratio for the entire area of tissue. The mean ratio was then determined for each group. The results for each group can be seen in Figure 3.

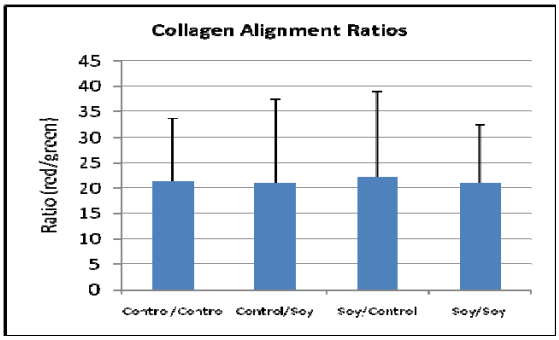

FIGURE 3

It is clear from the graph that a soy-based diet has no effect on the collagen alignment of the uterosacral ligaments. It can also be concluded from this data that the delivery of soy in the pre vs. post menopause period does not impact the effect soy has on the uterosacral ligaments.

DISCUSSION

Although the collagen alignment was unaffected the soy, it cannot be concluded that the collagen itself was unaffected. More studies are needed to corroborate these results. Also, there were some limitations to the study that may have had an impact on the collagen ratios. There was an insufficient amount of exposure time to the soy diet (2.5-5 years). Studies have shown that more than 10 years may be needed in order to observe any effects. In addition, collagen alignment may not have been an accurate predictor of tensile strength. A collagen subtyping of the ligament may be more appropriate. There was also a variability of the fibrous component of the uterosacral ligaments seen from the trichrome stain. This could account for some of the differences between the collagen alignment ratios and the high standard deviations among group. More studies are needed on the connective tissues of the pelvic floor in order to gain a better understanding of pelvic organ prolapse and how to prevent it.

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ACKNOWLEDGMENT

First I would like to thank my mentor Dr. Abramowitch for allowing me to work for his lab and letting me participate in his research. I would also like to thank Dr. Moalli for allowing me to participate in this primate study and teaching me during the primate surgeries. I also give my thanks to Suzan Stein for helping me with my project and while doing research. And finally I would like to thanks all of the MSRC staff for making this opportunity available for me.

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My story begins in the town of Frankfort, IL, a small suburb about an hour from the city of Chicago. As you might guess, Chicago is my favorite place in the world, and I have yet to visit another city that can match what it has to offer. However I still like to travel and explore, which is what led to me to attend the University of Pittsburgh. In the two years I have been here, I have really come to enjoy the city, the people, campus, and appreciate all the opportunities available.

To this day, I still play a variety of sports; my favorites include basketball, tennis, and running. However, these activities were merely hobbies, and I never thought my interest in them could result in a possible career choice. I entered college as a bioengineer and premed major, but had never really heard of the field of biomechanics. However, a lab visit to the MSRC sparked my interest in the field, and the realization that the engineering principles used at the lab could help greatly in a clinical context was enlightening. It dawned upon me then that doing research at a lab such as this one, would be very beneficial to guide my future career goals.

So far, the summer experience has not disappointed in the slightest. In my ten week stay, I have learned a great deal, not only on the topic of my research, but also how research at a university is handled and operated. In particular, I am impressed at the MSRC's ability to encompass a variety of different biomechanics researchers under one roof, and the coordination involved in making it work. I want to thank my mentor John, for being patient with me, and teaching me as much as possible in our short time together. I want to thank Dr. Woo, and the entire MSRC for taking me under their wing, and providing an excellent experience.

THE ALTERATION OF STRUCTURAL AND MECHANICAL PROPERTIES OF THE HUMAN PATELLAR TENDON DUE TO MULTIPLE FREEZE-THAW CYCLES

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Introduction: In recent years, tendon and ligament allografts have gained popularity and acceptance when performing anterior cruciate ligament (ACL) repair. A recent study comparing the post operative condition of patients who underwent ACL reconstruction using allograft vs. autograft tissue showed no statistically significant difference (patients were questioned about symptoms and subjected to detailed physical examination)[1] The advantages for this surgical procedure include decreased surgical morbidity, unchanged patellofemoral tracking, and lower costs.[2] However, the risks associated with such a procedure are great, and may result in disease transmission, including but not limited to Hepatitis B, C, Human Immunodeficiency Virus (HIV), and bacterial infections.[3] As a result, allograft tissue is subject to a battery of tests, screens, and passed through several processing agencies before reaching the operating room. To account for these conditions, the tissue is often in a frozen state, and thawed upon reaching the testing site, and can often undergo many of these freeze-thaw cycles.

A study completed by Park et. al analyzed the affects of cryopreservation (-80°C) on a rat bone-patellar tendon-bone block, and histomorpholgogy suggested that collagen fibers of these tendons were affected by ice crystal formation, and thus tensile testing of these tendons showed negative effect in structural and mechanical properties.[4] The purpose of our study is to focus examine the effects multiple freeze-thaw cycles (1,4, or 8) have on the structural and mechanical properties of the bone-patellar tendon-bone block, intended to simulate the various processing techniques of a patellar tendon allograft. Also, the viscoeleastic properties of the tendons in each freeze-thaw cycle are to be compared using a stress-relaxation test. Furthermore, the histology of the tendons are to be analyzed to determine the effects of the freeze-thaw cycle on collagen arrangement and distribution, as well as other biochemical factors. Our hypothesis is that the multiple freeze-thaw cycles will cause a significant change in the structural and mechanical properties of the bone tendon block.

Materials and Methods: 55 whole patellar tendon and tibial bone section specimens were obtained, which have only one known Freeze-Thaw cycle were enrolled in this study. The specimens were donated by the American Association of Tissue Banks(AATB). Upon harvesting, the patella tendon was first checked for any defects, such as open growth plates, hemorrhages or holes in the ligament, and cracks in the patellar or tibial bone block. For each specimen, the medial lateral tendon thickness was measured, and an approximately 10mm thick portion was excised, along with the corresponding patellar and tibial bone plugs.

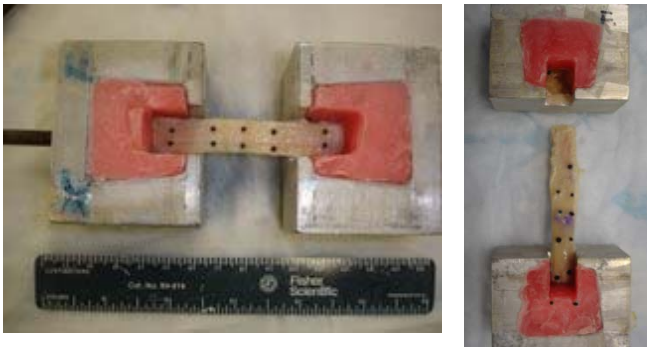


Figure 1: BPTB block in Instron Testing Jig

The tibial bone block was cut to fit the mechanical testing jig, approximately 2x1x1 cm. The parameters of the study called for a total of 55 BPTB specimens, and divided categories based on sex, age, and freeze-thaw cycle number. A total of 32 male and 23 female knee samples were obtained, containing the patella-patellar tendon-tibia bone block, as well as other surrounding tissue. The specimen age ranged from 27 to 85 years. Specimens were randomly assigned to either 1,4, or 8 freeze-thaw cycles, with the aim that an equal amount of specimens in each age group would be placed in each freeze thaw cycle. The specimens were obtained from the donor, and shipped frozen to the processing agency (AATB), then via dry ice to the MSRC. The specimens were thawed for testing, and this completed one freeze thaw cycle. Multiple freeze thaw cycles involved alternating between placing the sample in a freezer at -20±10°C for at least six hours, and thawing at 22±3°C for at least 90 minutes (up to six hours to simulate clinical conditions)**.** For samples whose medial-lateral patellar tendon thickness exceeded 24 mm, the tendon was divided in two, and each respective sample was placed in a different thaw freeze cycle, to allow for more accurate comparisons. After a sample had completed its assigned number of freeze-thaw cycles (1,4, or 8), hygienic orthodontic resin (Lang Dental, Wheeling, IL, U.S.A) was used to fix the sample to a machine jig (custom made at the MSRC). A laser micrometer was used to determine the cross sectional area of the tendon, as three readings were taken at the proximal (femoral), middle, and tibial (distal) side of the tendon, and an average of these readings was used as the reference value. Ten plastic reflective markers were evenly glued throughout the surface of the tendon and insertion sites for strain tracking (DMAS Motion Analysis), and a picture was taken of the specimen. The clamps containing the BPTB specimen were submerged in a 37 °C .9% saline solution, the tendon was slacked and allowed to equilibrate for 30 min. The clamps were fastened to the materials testing machine (Model 5565; Instron, Canton, MA, U.S.A), then tendon was checked for proper

alignment, and a video capture system was set up. At the start, the load was balanced, 2 N was applied to the system, and the gauge length was reset (set as 0 mm). Specimens were pretensioned at 89 N (20 pound force) and held in displacement control for 25 minutes. Tension was adjusted back to 89 N (20 pound force) at 5 minutes and 15 minutes. After 25 minutes, the specimen was slacked for 30 minutes before commencing cyclic loading. The test was completed exerting loads alternating between 50 and 250N for 100 cycles at 1 Hz, with an elongation rate of 20 mm/min. Tendon elongation (creep phenomena) was recorded for the first and last three cycles of the test. The specimen was slacked to 0N after the test was completed and allowed to sit for 30 min. Finally, a Load Failure Test was completed on each specimen, starting at 0N (and completed when 80% of the maximal load had been reached), with an elongation rate of 50 mm/min. The failure mode was recorded for each specimen. Statistical analysis for the specimens was conducted using SPSS. A statistically significant result was indicated by $p < .05$.

Results: The results of the study still in progress reveal interesting trends. A total of 28 tendon samples were tested. Eleven underwent 1 freeze-thaw cycle, ten underwent 4 freeze-thaw cycles, and seven underwent 8 freeze-thaw cycles. The ultimate load and stiffness for each sample were recorded and compared among each freeze thaw cycle. The results are illustrated in the figure below.

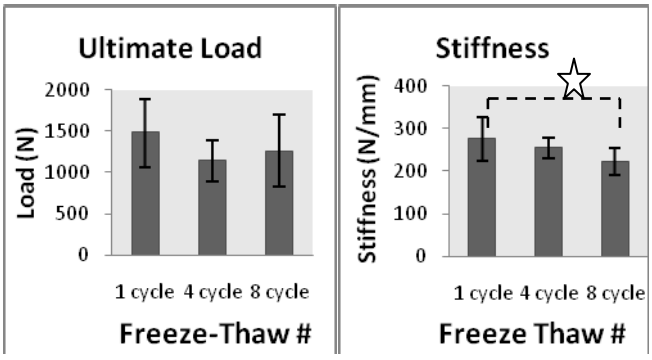
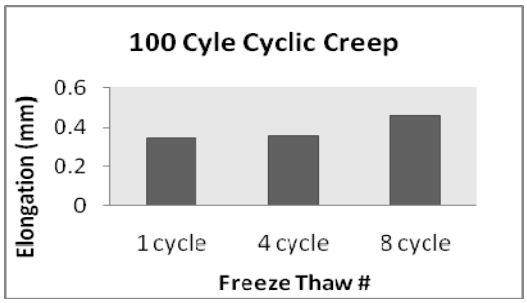


Figure 2: Structural Properties Data for all Specimens

A non parametric statistical test was run, and no significant difference was found in ultimate stiffness between the samples. However, a significant difference(☆) was found in the stiffness between 1 and 8 freeze-thaw cycles as p=.028. A graph was also recorded analyzing paired specimen data, in which tendons from the *same knee* were placed in different freeze thaw cycles, and the compared. This reduced the variables between samples, and allowed for more accurate comparisons. Due to the sample size (n=5), a parametric test was done, and no statistically significant difference was found in neither ultimate load nor stiffness. Cyclic creep data was also analyzed for a 100 cycle test for 1,4,and 8 freeze-thaw cycles. The results are indicated in the figure.



	1 TF cycle (mm)	4 TF cycles (mm)	8 TF cycles (mm)
100 cycle test	0.34 ± .22	0.36 ± .15	0.46 ± .25

Figure 3: 100 cycle creep data

Finally, the failure mode, either substance tear or bony avulsion, was recorded for each of the 28 samples tested, and compared among each of the different freeze-thaw cycles. 15 failures occurred via substance tear, and 13 occurred via bony avulsion.

Discussion: Although the study has not been completed, there are some interesting trends to observe. First, a statistically significant difference was spotted in stiffness between the 1 and 8 freeze-thaw cycle groups. However, this difference was not spotted between paired specimen data, a more accurate comparison. This could mean the significant difference found was due to a result of physical differences between tendons of different knees (length, thickness cross-sectional area, etc.). However more samples will be needed for paired specimen analysis to draw further observations. A primary goal of the study was to determine not only the structural properties, but also the mechanical properties of these tendons. A problem was encountered in calculating the mechanical properties due to the variability of the markers placed on the tendon. The complex tears encountered during the load failure test made it difficult to use consistent markers in calculating the strain rate, and ultimately, strain. Also, failures that occurred by bony avulsion made it impossible to calculate the *true* mechanical properties of the tendon. In future work, a consistent marker set will need to be established to acquire the mechanical properties. The failure mode recorded showed no significant differences. It was previously hypothesized that freeze-thaw cycles might deteriorate bone quality as well as the tendon, which might result in more bony avulsions. However, more samples will be needed to evaluate this hypothesis.

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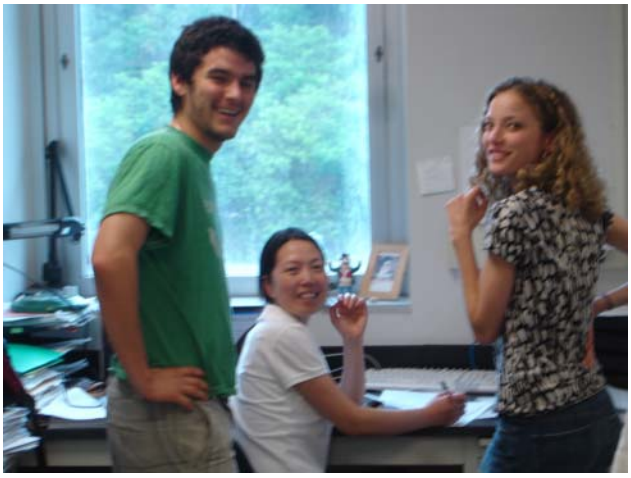

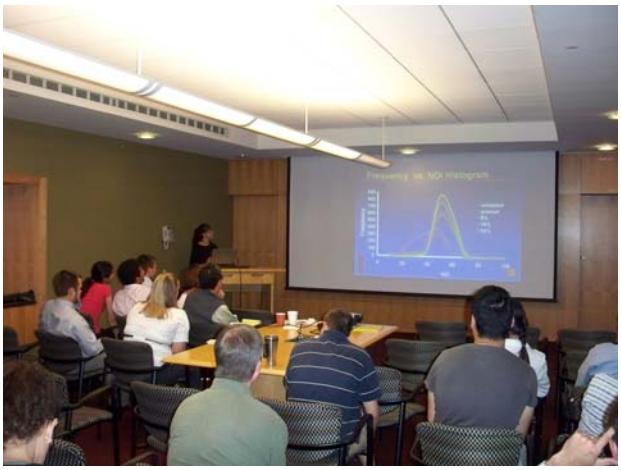

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Summer 2009

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